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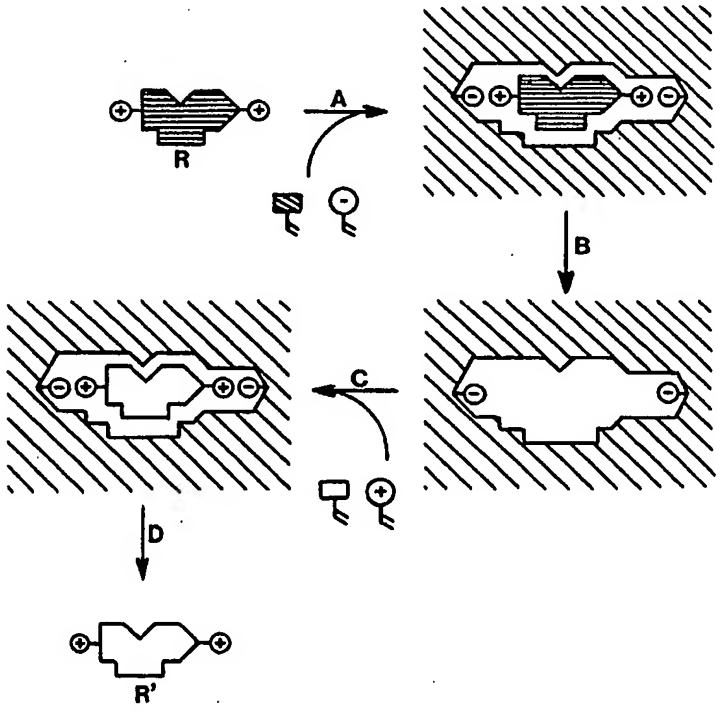
(54) Title: PREPARATION AND APPLICATION OF ARTIFICIAL ANTI-IDIOTYPIC IMPRINTS

(57) Abstract

This patent application describes the use of molecular imprinting as a means for preparation of anti-idiotypic imprint matrices. With this technique, imprints of artificial or natural molecules including their recognition sites, such as of molecularly imprinted polymers or biological receptors, antibodies or enzymes, can be prepared. In the former case, using the original imprints as casts or molds in a subsequent polymerisation step, utilising preferentially functionally complementary monomers, the formation of imprint materials containing recognition sites complementary in shape and functionality with the original imprint can be obtained but being of different composition. Alternatively, imprints or images of the artificial or biological species can be obtained directly. The so formed preparations can be used in a vast variety of applications, e.g. as new drugs, inhibitors or new affinity materials.



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PREPARATION AND APPLICATION OF ANTIFICIAL ANTI-IDIOTYPIC IMPRINTS

The present invention concerns the preparation and application of artificial anti-idiotypic antibodies obtained by molecular imprinting.

It is known from Nature that antibodies can in their turn give rise to anti-antibodies. Such anti-antibodies or anti-idiotypic antibodies have been investigated widely [1]. The combining site of an anti-idiotypic antibody may display structural features which may be the "internal image" of the original antigen (the previous antibody). Attempts have also been described lately of producing monoclonal anti-idiotypic antibodies being functional internal images of enzyme active sites leading in one case to the formation of a catalytic antibody with cholinesterase activity [2]. This was carried out by allowing an antibody raised against an enzyme, i.e. cholinesterase, to be injected resulting in the aforementioned anti-antibodies.

A more presently developed technique, that of molecular imprinting, is a major element for the here described invention [3, 4]. It is the name given to a process for preparing polymers that are selective for a particular compound (the print molecule). The technique involves: (1) prearranging the print molecule and the monomers and allowing complementary interactions (non-covalent or reversible covalent) to develop; (2) polymerising around the print molecule-monomer complex; and (3) removing the print molecule from the polymer by extraction (figure 1). Polymerisation thus preserves the complementarity to the print molecule and the polymer will selectively adsorb the print molecule subsequently. The technique has also been referred to as "host-guest" polymerisation or template polymerisation.

The invention will now be described further with reference to the accompanying drawings, in which:

Fig. 1 shows the principle of molecular imprinting. Development of complementary interactions between the print molecule and the monomers (a); polymerisation (b); removal of the print molecule from the polymer (c). M, monomers; PM, print molecule; CR, crosslinker.

Fig. 2 shows the principle of the invention in one of its aspects.

Fig. 3 shows a second aspect of the invention.

Fig. 4 shows a third aspect of the invention.

Fig. 5 shows a forth aspect of the invention.

Fig. 6 shows the preparation of an anti-idiotype imprint, preferentially with a further polymerisation (imprinting) system, corresponding to 4 in Fig. 4.

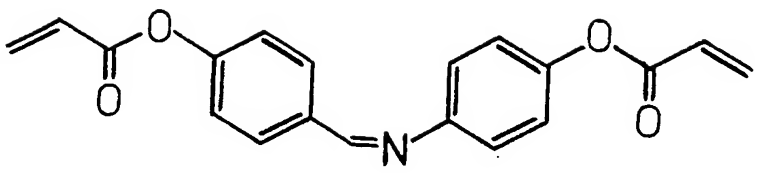
It has been demonstrated that molecular imprints can serve as mimics of naturally occuring binding sites [5]. Furhermore, such imprints can also be made to form recognition sites of a wide range of compounds. Analogous to anti-idiotype antibodies, the obtained cavities of such original imprints can be used as molds for complementary "daughter imprints" leading to anti-idiotypic imprints or images of the original imprints. The resulting images would resemble the original imprint species in shape and functionality. The principle is schematically described in Fig. 2.

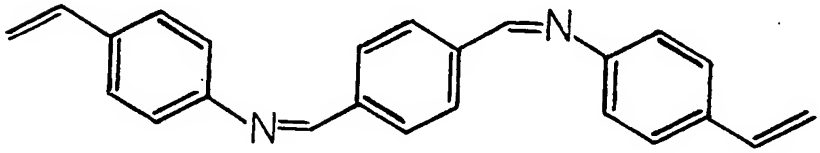
A molecular imprint is made against a compound, e.g. compund R carrying positive chages, using monomers functionally complementary to the print molecule, e.g. monomers containg negative charges, in step A. The cavities obtained after removal of the imprint molecule (B) are subsequently filled with other complementary functional e.g. polymerisable building stones, e.g. positively charged monomers, and polymerisation is allowed to take place (C). In the following step the original cast or mold which carried the original imprint is removed (D) leaving the anti-idiotypic imprint or imprint of the original

imprint free. The obtained molecule, R' which could be called "filler", should resemble or be complementary to the original imprint molecule in shape and functionality, but is, dependent on the "filling material" applied, different in composition than R.

As an extension of the technique one can envisage, what could be called surface-imprinting, the following: (Fig. 3). A molecular imprint is made against a compound, e.g. compound X carrying positive charges. A functionalised matrix (M) carrying polymerisable groups together with complementary monomers, e.g. negatively charged monomers, are utilised in the polymerisation step A. Extraction of the print molecule (B) leaves the polymer with recognition sites complementary in shape and functionality to compound X. Using these sites as molds or casts in a kind of anti-idiotype polymerisation utilising complementary monomers, e.g. positively charged monomers, (C) and subsequent removal of the first polymer (D) renders an anti-idiotype imprint polymer (Y) mimicking the original compound X. Thus, the surface-imprint of the original molecule (X), is prepared which could be employed to create the structurally related molecule (Y). This technique should be especially useful for larger molecules.

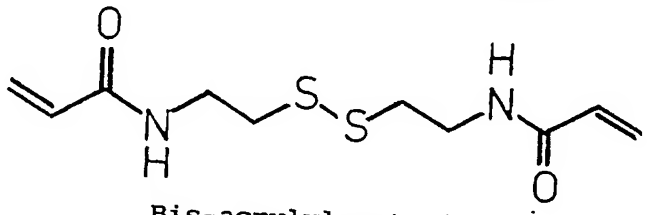
One requirement for the envisaged imprint is that both the original matrix as well as the subsquently obtained matrix are capable of allowing imprints to be made. Another requirement is that the original, i.e. the first imprint material, can be removed without interfering with the memorising capability of the second imprint. A number of possible materials and approaches are given in the examples. These include the use of reversibly crosslinking monomers such as the use of crosslinkers containing Shiff's base-linkages [6]. The latter polymers are easily dissolved.





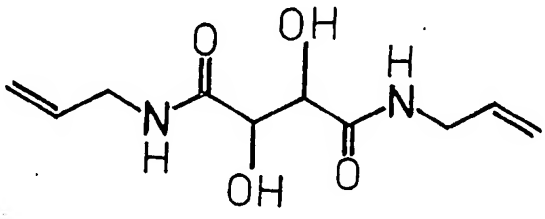
Crosslinkers containg Shiff's base-linkages.

Another possibility is the use of disulfide containing analogs of bis-acrylamide, e.g. bis-acrylylcystamine, which can be dissolved with 2-mercaptoethanol [7].

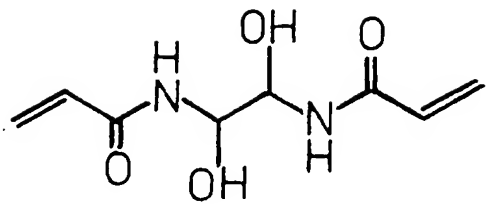


Bis-acrylylcystamine

Other crosslinkers that can be cleaved are N,N'-diallyltartardiamide [8] or N,N'-(1,2-dihydroxyethylene)bisacrylamide [9].



N,N'-diallyltartardiamide



N,N'-(1,2-Dihydroxy)-bisacrylamide

Another possible way is to use completely different matrices as first or second imprints such as agarose or silica, the latter prepared by polymerisation of silanes. As an alternative to the removal of the first imprint by subsequent dissolution of the matrix, the second imprint may be loosened up using such reversibly dissolving monomers whereby care is to be taken to protect the recognising properties of the second imprint matrix. Other approaches facilitating such dissociation include the use of magnetic beads carrying the imprint molecules. Such matrices can, posterior to polymerisation, be separated by application of a magnetic field forcing the matrices apart. Another alternative approach involves the polymerisation at interfaces.

An additional alternative way to obtain imprints would be to directly make imprints of biomolecules by filling the active or binding sites of the latter with monomers and subsequently using the formed matrix as a mold or cast in a second imprinting step. This would lead to the formation of anti-idiotype imprints of the first preformed imprints as depicted in Fig. 4. The particular binding species is immobilised on a degradable matrix (1), e.g. agarose beads, and a polymerisation mixture containing functionally complementary monomers is added in step A. Following polymerisation the original matrix is dissolved in step B making the newly formed polymeric anti-idiotype binding sites (3) accessible. Such preparations could then be used e.g. as artifical inhibitors or receptors either while arranged as a film or thin membrane or

in smaller units. Further, as indicated in Fig. 4, one can go from 3 over step C employing the imprint of 3 to obtain via step D a plastic imprint 4 similar to 1.

Further, as an alternative to the above direct imprinting, one can envisage the lining up of monomers or other molecules along a surface or active site of e.g. biomolecules as outlined in Fig. 5. The former are allowed to interact with functional groups of the molecule, 1, in step A followed by their condensation (B). Removal of 1 in step C leads to the formation of a thin-layer imprint of 1. Alternatively, the functional groups of the biomolecule are first derivatised followed their condensation.

Potential use of such imprints (= filler molecules)

(1) They could lead to new enzyme inhibitors, new drugs, new affinity ligands, new anti-affinity material including cell-affinity material, anti-antibodies and new catalysts.

(2) They could replace peptides, nucleotides, carbohydrates or other biological material with other material such as organic polymers.

(3) They could lead to new compunds with identical or similar function as the imprint species but being more stable and cheaper to prepare.

(4) In an extension, in cavities or on surfaces obtained by imprinting new enzyme-like catalysts may be obtained.

(5) Such cavities if they are chiral can be used as scaffold to create new chiral molecules.

(6) The cavities obtained from the first imprint can be used to create new molecules utilising combinatorial libraries of various organic or inorganic molecules.

(7) Analogous to anti-idiotype antibodies.

Example 1

Acetylcholin can be molecularly imprinted using a modified standard protocol [10]. Thus, acetylcholin is polymerised with methacrylic acid, different types of crosslinkers including those that can be selectively split (e.g. bis-acrylylcystamin) and initiator (azo-isobutyronitrile, AIBN) in chloroform. The formed imprints are complementary in shape, charge and hydrogen bonding capabilities to acetylcholin. The imprint molecule is extracted from the polymer using methanol/acetic acid (9:1). Susquently, a cocktail of small polymerisable monomers with or without crosslinkers is added in the formed cavity and allowed to polymerise. Addition of agents to dissolve the formed original matrix, such as SH-carrying compounds, make the daughter imprints become accessible. The binding capabilities of the obtained anti-idiotype analogues of acetylcholin could be tested in competition assay against free acetylcholin.

Example 2

A molecular imprint against the trypsin inhibitor p-aminobenzamidin is prepared. The imprint species is polymerised with appropriate monomers, e.g. methacrylic acid and breakable crosslinkers, e.g. bis-acrylylcystamine. Following extraction of p-aminobenzamidin, an anti-idiotype imprint is made using the p-aminobenzamidin imprint material as a cast in a new polymerisation step. The original imprint material is dissolved leaving the mirror imprint material free for application. The formed anti-idiotype imprint material, mimicking the trypsin inhibitor, can subsequently be analysed in an affinity assay against trypsin or in a competition assay against large trypsin inhibitors like bovine pancreatic trypsin inhibitor (BPTI).

Example 3

Trypsin is immobilsed on agarose beads. Anti-idiotype imprints of trypsin are prepared similar to the preceding examples by polymerisation of an appropriate monomeric cocktail and suitably crosslinkers on the surface of the agarose beads. Addition of an acidic solution leads to dissolution of the agarose beads thus making the anti--idiotype imprints accessible. Analysis can be perceived using the anti-idiotype imprint material as an affinity matrix for trypsin.

Example 4

A silicon surface or microslide was first oxidised and then silanised with aminopropyl triethoxy silane in aqueous solution overnight. The slides were then treated overnight with succinic anhydride at pH 6 which leaves only free carboxyl groups at the surface. In the next step the slides were extensively washed with dioxane to obtain anhydrous conditions and reacted with N-hydroxysuccinimide and N,N'-dicyclohexylcarbodiimide in dioxan for 2 h which converts the carboxyl groups to N-hydroxysuccinimid esters. After further washing, the slides were treated with insulin in 0.1 M $NaHCO_3$ buffer pH 9.2 overnight. Under these conditions the amino group of lysine B29 react specifically with the N-hydroxysuccinimide ester. Alternatively the coupling could be achieved at pH 6.4 via the terminal amino groups of B1 phenylalanine.

Determination of the amount of bound insulin was carried out by ellipsometry.

Insulin possesses a surface, zinc binding histidine at B10 on the opposite side of the structure from the immobilisation site (B29) (B1 et al, Biopolymers 23 (1984), 391-395). There are five other basic amino acids at the surface which are positively charged below the isoelectric point of 5.5 and will associate with sulphonate groups (Matsuura et al., JACS 115 (1993), 1261-1264). Thus, imprinting can be carried out with i) an aqueous

solution of the chelating monomer N-(5-methacrylamido-1--carboxypentyl)iminodiacetic acid, $CuSO_4$, styrene sulphonate, acrylamide and piperazine-bisacrylamide at pH 4 and 5°C using methylene blue as photoinitiator, or ii) an organic solution of N-(4-vinyl)-benzyl iminodiacetic acid (VBIDA), $CuSO_4$, methacrylic acid (MAA) and ethylene glycol dimethacrylate (EGDMA) and using AIBN as initiator.

The imprinting forms a stable film, which can be separated from the surface on which it has been formed, e.g. manually or by dissolution.

The immobilised insulin was shown to promote lipogenesis by fat cells in a bioassay.

Example 5

According to similar processes as described in Example 4, penicillin G was coupled to a microslide by

a) Acrylate derivatisation. A sol of 1 Wt % of 3-(triethoxysilylpropyl)methacrylate in dry acetone is allowed to react with the slides by established procedures,

b) Amino derivatisation. To wet slides are added amino silane sol (10 % aminopropyl triethoxysilane in H_2O - pH adjusted to 3.5). 8.8 mg penicillin G, 25 mg EDAC, 25 ml (P) pH 7.0 are dissolved, left at room temperature for 30 min, followed by addition of the amino slides (4 per Petri dish, spread out), 2 ml penicillin G-coupling sol is added to each slide, ensuring that it covers the slide completely, and left overnight at room temperature.

Example 6

According to a process similar to the one described in Example 5, theophyllin was coupled to microslides.

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CLAIMS

1. A method of producing a mimic of an artificial or native molecule, c h a r a c t e r i s e d in polymerising in a first step monomers and crosslinkers in the presence of the molecule, thereby forming a matrix with an imprint of the molecule, separating the molecule from the matrix, polymerising in a second step monomers and crosslinkers, which could be the same as or different from the monomers and crosslinkers in the first step, in the imprint in the matrix, thereby creating a mimic of the molecule having the same functionality and/or shape as the molecule, and then separating the mimic from the imprint in the matrix.

2. A method of producing a mimic of surface bound molecules, c h a r a c t e r i s e d in polymerising in a first step monomers and crosslinkers under the formation of a film having imprints of the surface bound molecules, separating the imprinted film, polymerising in a second step monomers and crosslinkers, which could be the same as or different from the monomers and crosslinkers in the first step, on the imprinted film, thereby creating a mimic of the surface bound molecules with the same functionality and/or shape as the surface bound molecules, and then removing the imprinted film from the mimic.

3. A method of producing a mimic of surface bound molecules, c h a r a c t e r i s e d in polymerising monomers and crosslinkers under the formation of a film having imprints of the surface bound molecules, and then separating the imprinted film from the surface bound molecules.

4. Use of the method according to any one of claims 1-3 for the preparation of anti-idiotype like imprints of moieties comprising artificial or natural binding sites, such as molecular imprints, receptors, antibodies, enzymes and others, which anti-idiotype like imprints have the same functionality and/or shape as the moieties.

5. Use of molecular imprint for the preparation of anti-idiotype like molecules by the polymerisation of added monomers and crosslinkers within cavities made by molecular imprinting of moieties comprising artificial or natural binding sites, whereby the added polymerisable monomers and crosslinkers orient themselves within the sites prior to polymerisation.

6. Use according to claim 5, wherein the polymerisable monomers are acrylic monomers, silanes, or other interconnecting molecules, such as azides.

7. Use of molecular imprinting for the preparation of anti-idiotype like imprints for preparing new molecules by their direct formation in or around biomolecules or other molecules utilising preferentially polymerisable monomers and crosslinkers.

8. Use of such anti-idiotype like imprints as prepared according to any one of claims 4-7 as new inhibitors, new drugs, new affinity ligands, new anti-antibodies and new catalysts.

9. Use of molecular imprintingh for the preparationn of anti-idiotype imprints to create chirality within chiral cavities from non-chiral basic elements or using chiral monomers.

10. Use of a mimic produced according to claim 3 as a new inhibitor, new drug, new affinity ligand, new anti--affinity material including cell-affinity material, anti--antibodies and new catalysts.

11. Use of a molecular imprint, which imprint has been prepared by polymerising monomers and crosslinkers in the presence of a molecule, thereby forming a matrix with an imprint of the molecule, and then separating the molecule from the matrix, as a new inhibitor, new drug, new affinity ligand, new anti-affinity material including cell-affinity material, anti-antibodies and new catalysts.

12. Method of producing an entity complementary to a molecule, c h a r a c t e r i s e d in adding to the molecule, comprising active or binding sites, small cross-

linkable moieties having active groups complementary to the sites on the molecule, whereby the small moieties associate with said sites, thereafter crosslinking the small moieties associated with these active sites under the formation of an entity having active groups complementary to the molecule, and then separating the entity from the molecule.

FIG.I

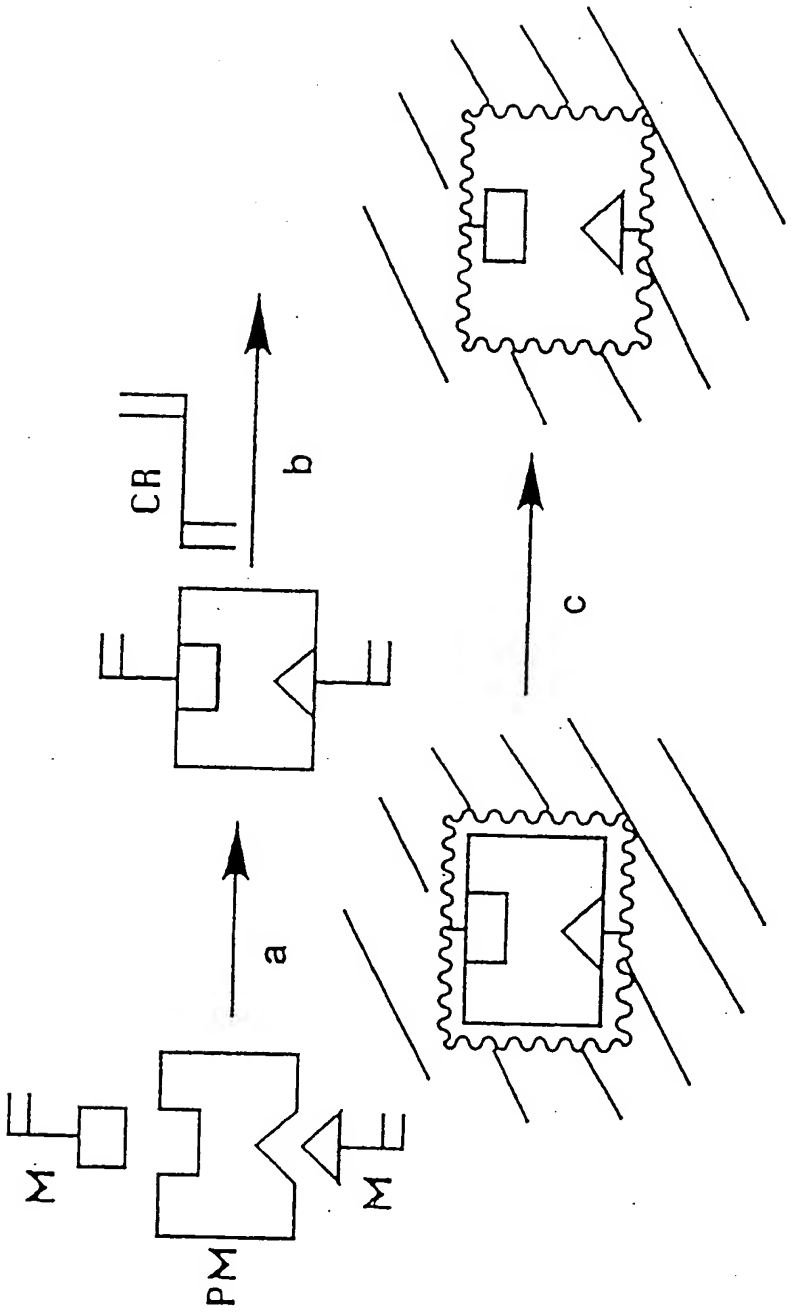

FIG.2

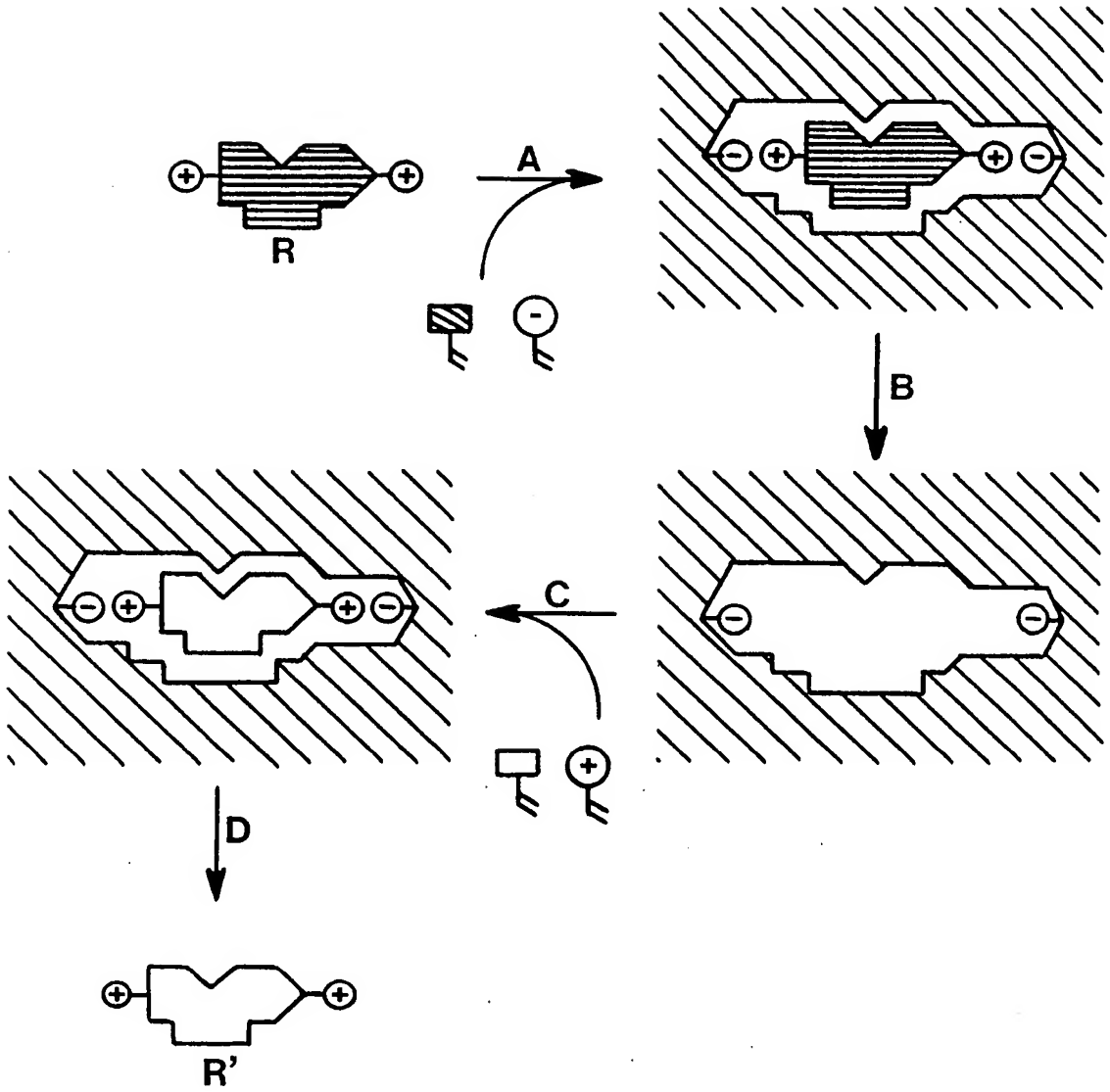

FIG.3

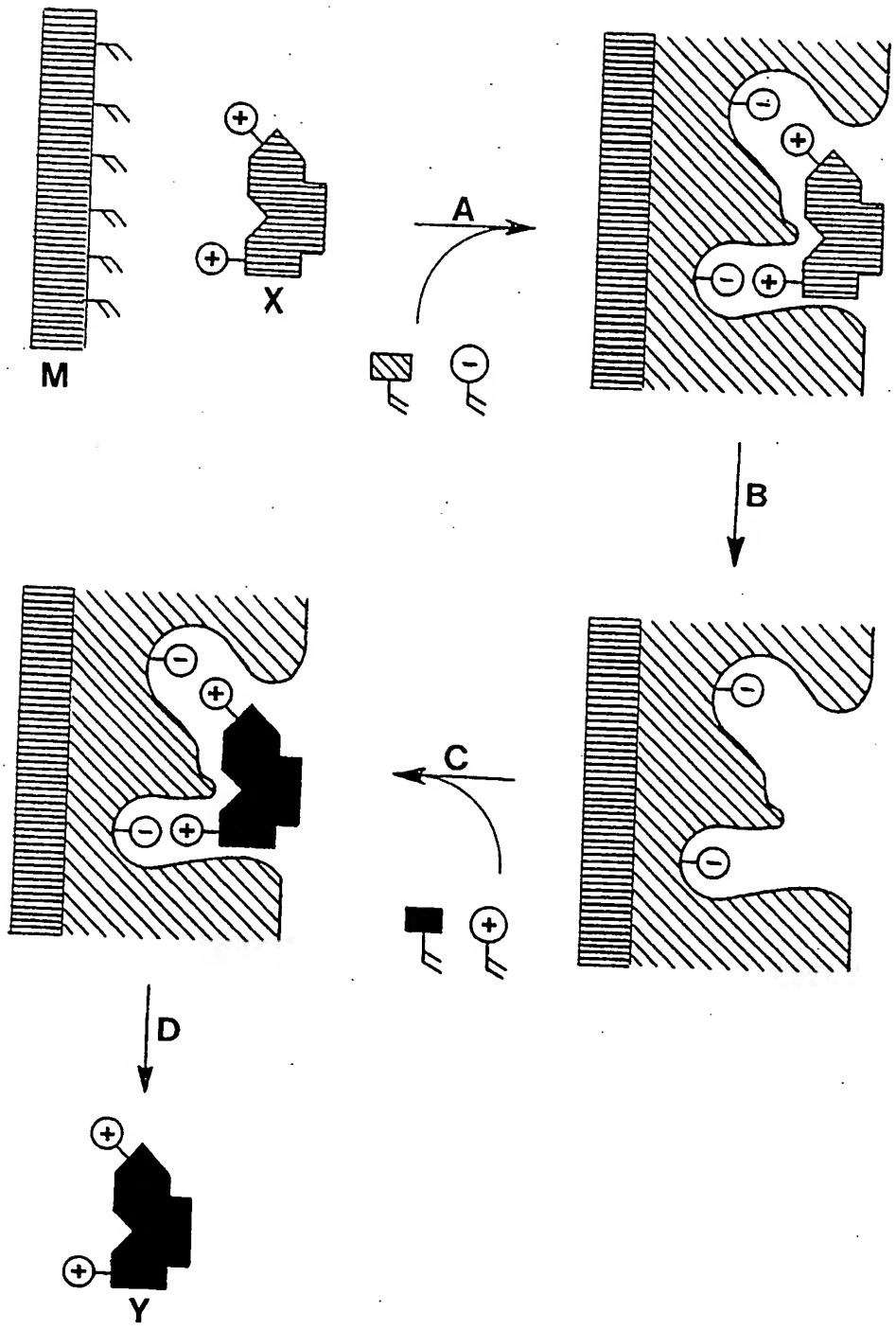

FIG. 4

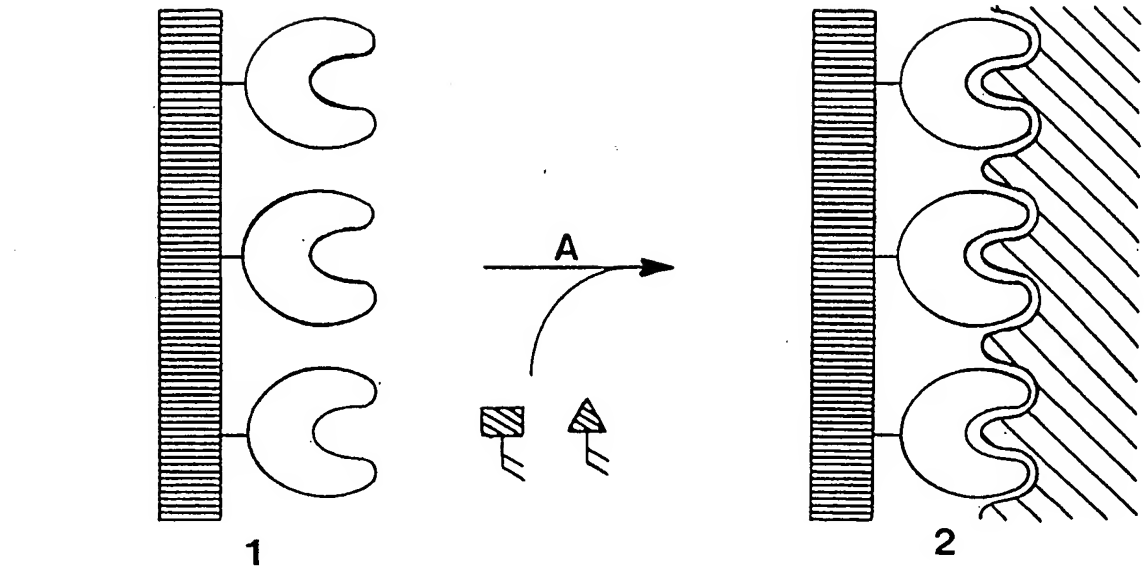

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FIG.5

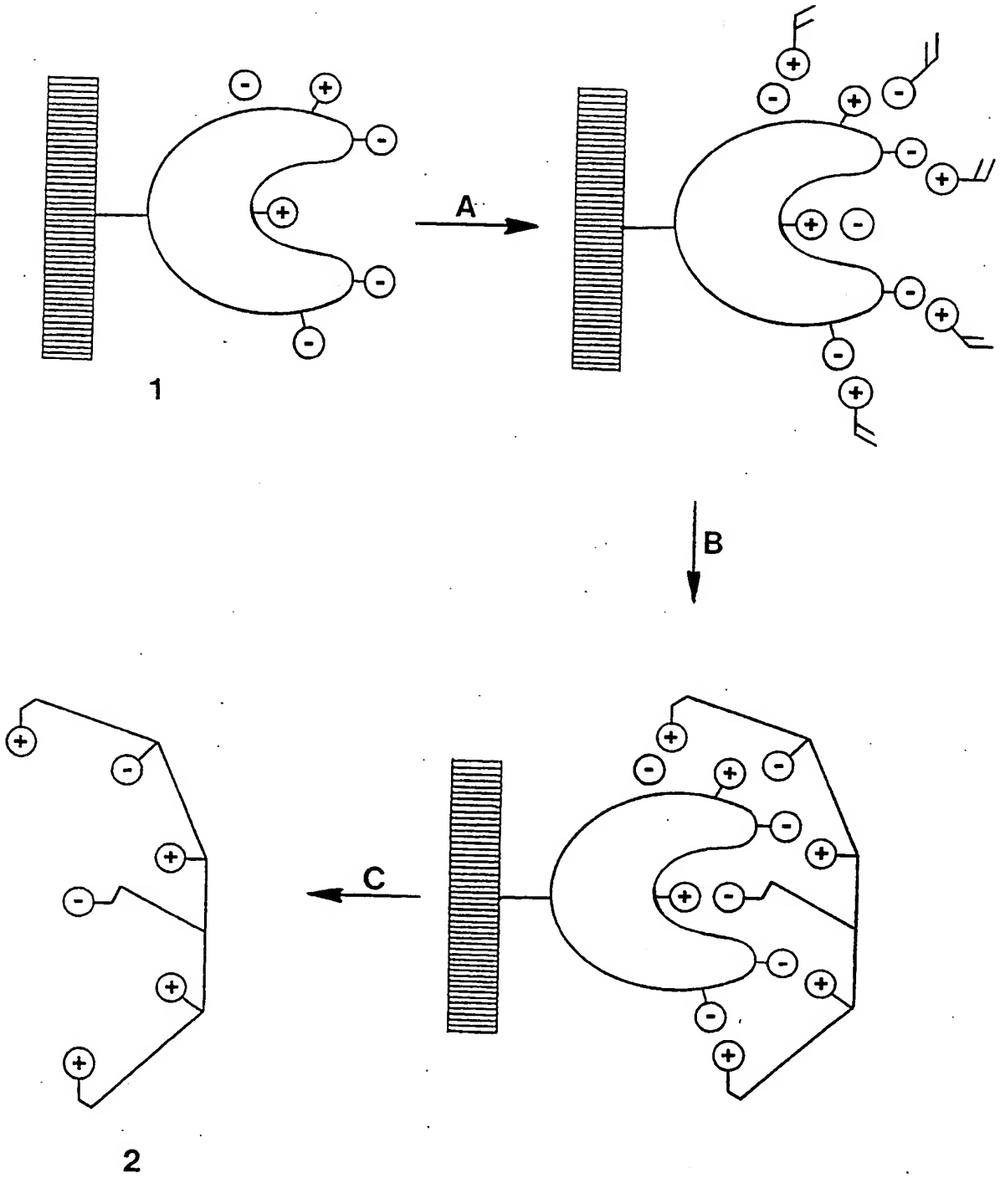
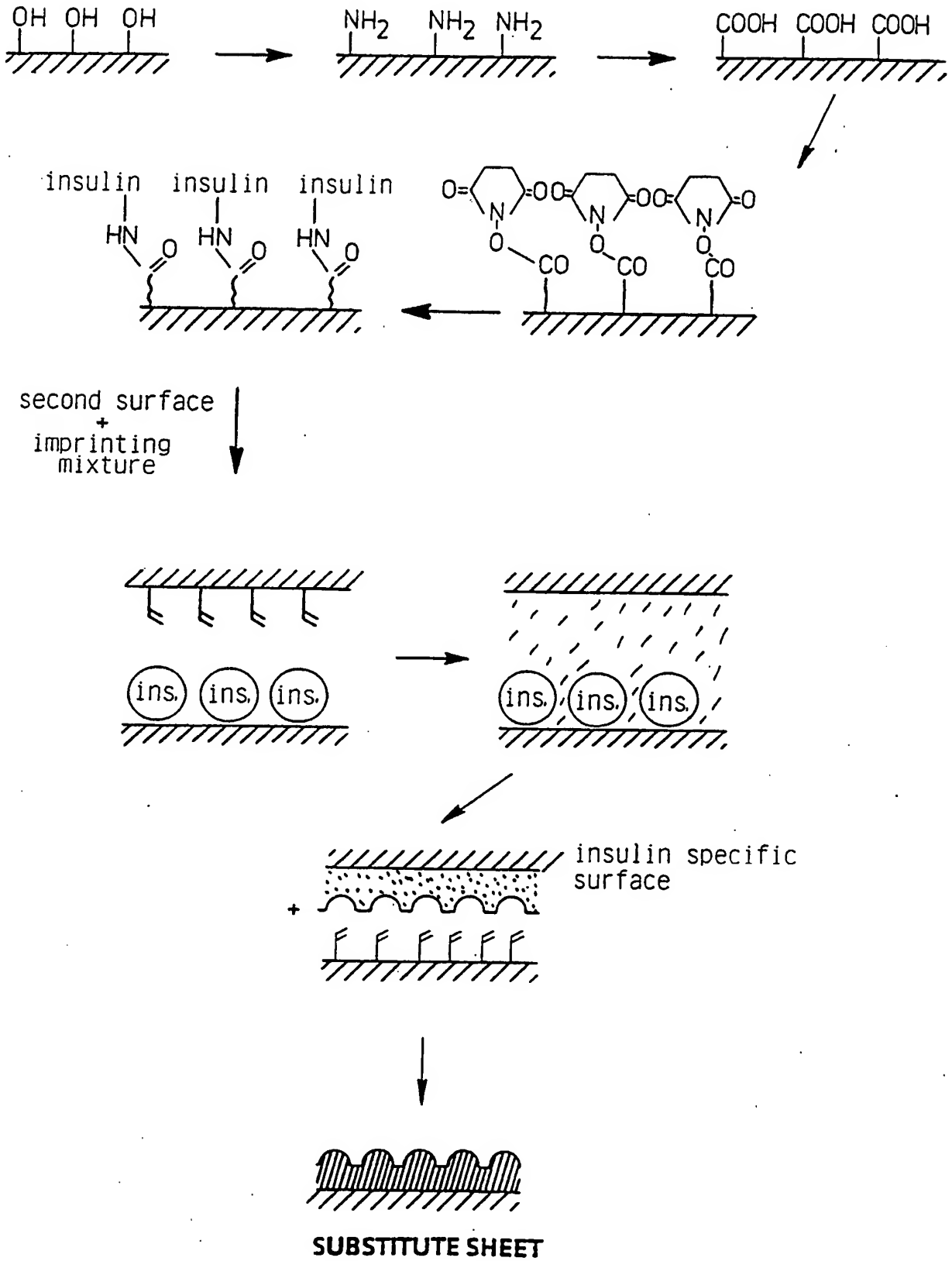


FIG.6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surface
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 95/00135

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: B01D 15/08, C07K 16/00, A61K 39/00 // G01N 33/53
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B. FIELDS SEARCHED

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

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because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see extra sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-2, 4-9 and 11

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

03/05/95

International application No.
PCT/SE 95/00135

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 5110833	05/05/92	SE-A- 8900130	16/01/89

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 95/00135

The present invention pertains to molecular imprints of imprints (anti-idiotypic imprints) and use of the imprints. Secondly a method is refered to in claim 3 which is a more general method of making an imprint of surface bound molecules. A further method according to claim 12 produces a complementary "entity" to a molcule comprising active binding sites. Small crosslinkable "moieties" having "active groups" complementary to the sites of the molecule are addes to the molecule and crosslinked after having been able to associate with the active sites. None of these inventions seems to be linked to each other so as to form a single inventive concept. Thus, the present claims do not conform with the requirements of unity of invention and refer to three inventions:

1. Imprinting of molecular imprints and use thereof according to claims 1,2,4-9 and 11.

2. Making an imprint of surface bound molecules according to claim 3.

3. Method of producing an "entity" as explained above according to claim 12.

Inventions 2 and 3 have been covered by the search to the extent they refer to imprints of an imprint and use thereof.